The Journal of Experimental Biology 214, 3286-3293

doi:10.1242/jeb.060509

RESEARCH ARTICLE

Roles of biogenic amines in regulating bioluminescence in the Australian glowworm *Arachnocampa flava*

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Accepted 6 July 2011

SUMMARY

The glowworm *Arachnocampa flava* is a carnivorous fly larva (Diptera) that uses light to attract prey into its web. The light organ is derived from cells of the Malpighian tubules, representing a bioluminescence system that is unique to the genus. Bioluminescence is modulated through the night although light levels change quite slowly compared with the flashing of the better-known fireflies (Coleoptera). The existing model for the neural regulation of bioluminescence in *Arachnocampa*, based on use of anaesthetics and ligations, is that bioluminescence is actively repressed during the non-glowing phase and the repression is partially released during the bioluminescence phase. The effect of the anaesthetic, carbon dioxide, on the isolated light organ from the present study indicates that the repression is at least partially mediated at the light organ itself rather than less directly through the central nervous system. Blocking of neural signals from the central nervous system through ligation leads to uncontrolled release of bioluminescence but light is emitted at relatively low levels compared with under anaesthesia. Candidate biogenic amines were introduced by several methods: feeding prey items injected with test solution, injecting the whole larva, injecting a ligated section containing the light organ or bathing the isolated light organ in test solution. Using these methods, dopamine, serotonin and tyramine do not affect bioluminescence output. Exposure to elevated levels of octopamine *via* feeding, injection or bathing of the isolated light organ indicates that it is involved in the regulation of repression. Administration of the octopamine antagonists phentolamine or mianserin results in very high bioluminescence output levels, similar to the effect of anaesthetics, but only mianserin acts directly on the light organ.

Key words: dopamine, tyramine, serotonin, octopamine, neuroanatomy, light organ.

INTRODUCTION

Among arthropods, the ability to bioluminesce has been identified in crustaceans, insects and myriapods (Harvey, 1952). All light-producing reactions investigated in arthropods utilise a chemical reaction known as the luciferin–luciferase bioluminescence reaction (Viviani, 2002). The luciferase within different phylogenetic groups is chemically different, suggesting that bioluminescence systems have evolved independently in a number of groups. Fireflies (Coleoptera) and northern krill (*Meganyctiphanes norvegica*) are two of the better-characterised arthropod bioluminescence systems. In both, biogenic amines are involved in bioluminescence regulation.

In northern krill, serotonin is the primary regulatory biogenic amine (Fregin and Wiese, 2002; Kronstrom et al., 2007). Serotonergic neurons innervate muscular sphincters that regulate the access of haemolymph to the light organ, thus serotonin regulates the flow of oxygenated haemolymph to the light organ, modulating the bioluminescence reaction (Kronstrom et al., 2007). In fireflies, regulation of oxygen supply to the light organ also seems to be the key regulator of bioluminescence. Octopaminergic neurons innervate the tracheal supply: a pulse of octopamine releases nitric oxide, which activates the flash reaction by allowing penetration of oxygen (Trimmer et al., 2001). Injection and infusion of octopamine into the haemolymph increases the intensity of bioluminescence (Christensen and Carlson, 1982; Hashemzadeh et al., 1985). In *Photuris* fireflies, the paired light organs are innervated by nerves extending from four octopaminergic dorsal unpaired neurons within the terminal ganglion (Christensen et al., 1983). The nerves do not innervate the photocytes directly, but rather synapse with the trachea constituting the reflector of the firefly light organ (Peterson, 1970). In the larval firefly light organ, which has an aposematic rather than sexual communication function, the photocytes are directly innervated, perhaps related to the fact that larvae glow rather than flash and indicating that innervation of the tracheal end cells of adult light organs is an adaptation allowing rapid, intense flashing (Oertel et al., 1975). A common feature of the light organ of the adult firefly and the northern krill is that they both have an extensive tracheal supply, suggesting that oxygen availability is vital for the production of bioluminescence.

Bioluminescence has evolved several times among Diptera (Sivinski, 1998), the brightest glowing being members of the Keroplatidae, including a single species from North America, *Orfelia fultoni*, and species of the genus *Arachnocampa*, found only in Australia and New Zealand (Baker et al., 2008; Baker, 2010). Both groups use bioluminescence to attract flying insects, which are ensnared in sticky silk and mucus traps constructed by the relatively immobile larvae (Fulton, 1941; Meyer-Rochow, 2007). Despite the functional similarity, the light organs of *O. fultoni* and *Arachnocampa* are substantially different and are not homologous. In *O. fultoni*, light is emitted in several segments of the worm-like larva through a secretion from ‘black bodies’, which are cells containing dark granules (Bassot, 1978). In *Arachnocampa*, the larval light organ is derived from the distal

segments of the four Malpighian tubules (Wheeler and Williams, 1915).

The *Arachnocampa* light organ has been described at the structural and ultrastructural levels. Most studies have focused on *A. luminosa*; however, no structural differences among species have been noted. The light organ, composed of the terminal cells of the four Malpighian tubules (Gatenby, 1959), is located in a swollen posterior segment. Externally, a tracheal reflector surrounding the light organ is visible through the cuticle. Gatenby described a single nerve extending posteriorly from the seventh abdominal ganglion (terminal ganglion), sending neural processes to the region of the light organ (Gatenby, 1959). An ultrastructural investigation reported fine nerves containing opaque and clear synaptic vesicles, characteristic of biogenic amines, running alongside the cells of the light organ (Green, 1979a).

A number of observations and physiological experiments have pointed to the nature of the neural regulation of bioluminescence in *Arachnocampa*. Larvae bioluminesce in long bouts during which their light output can be modulated (Broadley and Stringer, 2009). In the dark zone of caves, larvae of *A. tasmaniensis* maintain strong diurnal rhythmicity through a visually mediated synchronisation mechanism (Merritt and Clarke, 2011). Larvae douse in response to stimuli such as light and loud noise, usually over a period of 1–2 min (Gatenby, 1959; Broadley and Stringer, 2009). When stimulated by the presence of prey, they brighten (Broadley and Stringer, 2009). The bioluminescence output comes under circadian regulation as well, with *A. flava* larvae maintaining rhythmic bioluminescence under constant darkness (Merritt and Aotani, 2008). Larvae in forest habitats tend to glow most brightly after dusk and slowly diminish towards dawn. Control of light production by the nervous system was indicated by ligation experiments whereby separation of the light organ from the rest of the body posterior to the terminal ganglion caused the light organ to spontaneously produce bioluminescence, suggesting that neural connections between the terminal ganglion and the light organ are required to repress light output (Gatenby, 1959). One possibility is that a neural connection is required to restrict oxygen flow from the tracheal reflector to the light organ (Gatenby, 1959). Anaesthesia with ether, chloroform or carbon dioxide also causes a release of bioluminescence in *A. richardsae*, even in the presence of high ambient light levels (Lee, 1976). The response suggests that bioluminescence is actively repressed during the day and modulated at an intermediate level at night, further indicating that light output remains downregulated even during the active glowing period.

Here we examine the morphology of the cells of the light organ of *Arachnocampa flava* Harrison 1966 and use immunostaining to trace their innervation. Given the involvement of biogenic amines in regulating bioluminescence in other arthropods, the effect of exposure to candidate biogenic amines and their antagonists is investigated. Different means of introducing the amines are trialled, including feeding, injection of amine solutions into the whole larvae, injection into the terminal segment carrying the light organ and bathing the dissected light organ in amine solutions.

MATERIALS AND METHODS

Experimental animals

Arachnocampa flava larvae were collected from Springbrook National Park, Queensland, Australia. Larvae were kept in the laboratory at 23±1°C, under a natural day–night light cycle. Larvae were housed in individual inverted plastic containers with clay pressed into the base (Merritt and Aotani, 2008) and were fed a single vinegar fly, *Drosophila melanogaster*, once per week in the afternoon. The instar of field-caught larvae could not be determined without destructive investigation. The New Zealand species *A. luminosa* was reported to have five instars covering a total larval period of 1 year (Pugsley, 1980). In this study, medium to large larvae were collected, probably corresponding to the fourth and fifth instars. The larvae were acclimated after collection from the field for 1 week before commencing experimentation. Individuals were not fed for 7–8 days before being used in experiments. Abstinence from feeding for approximately 1 week is not considered to be a state of starvation for *Arachnocampa*, as it has shown that in the wild that *A. luminosa* capture prey every 5–37 days (Broadley and Stringer, 2001).

Treatment with biogenic amines

Vinegar flies were used as a vehicle to orally introduce biogenic amines or antagonists to individual larvae. Compounds tested were octopamine hydrochloride (O0250), serotonin hydrochloride (H9523), phentolamine hydrochloride (E5156), mianserin hydrochloride (M2525), epinastine hydrochloride (E5156), dopamine hydrochloride (H8502) and tyramine hydrochloride (T2879) (Sigma-Aldrich Pty Ltd, Sydney, New South Wales, Australia). Fixed volumes and concentrations of these solutions were injected into the thorax of chilled vinegar fly adults using a pulled glass capillary connected to a pressure injector (MPPI-2, Applied Scientific Instrumentation, Eugene, OR, USA) at an injection pressure of 70 kPa for a duration of 180 ms. The injection volume was calculated at 1.2±0.4 µl by injecting saline into a drop of oil on a microscope slide and calculating volume using the equation for the volume of a sphere. A single treated fly was then placed into the snare of each *A. flava* larva in the late afternoon, 0.5–1 h before commencement of the 12 h scotophase (dark cycle) using an artificial cycle of 12 h:12 h light:dark. Light output from the larvae was recorded through the ensuing 12 h using a digital Firewire camera (XCD-X710, Sony Australia, Ltd, Sydney, New South Wales, Australia). Individual frames were captured every 10 min over the 12 h of the dark cycle.

Direct injection of biogenic amines into larvae was used as an alternative method of introducing the amines into larvae. Octopamine (0.58 and 0.058 mol l^{-1}) was injected into the posterior half of a larva, taking care to avoid impaling the light organ. The volume of biogenic amine solution and the method of injection followed the protocol described above. As *Arachnocampa* larvae possess a hydrostatic skeleton, the internal organs tended to extrude following injection into the haemolymph, causing death within hours; thus this method was used only for octopamine treatment.

Larvae were ligated between the terminal abdominal ganglia and the light organ using silk thread. The larvae were then cut in two anterior to the silk thread to ensure that all neural connections between the light organ and the terminal abdominal ganglion were removed. Biogenic amine solutions were injected into the ligated segment. Treatments consisted of injection of 1.2±0.4 µl of 0.58 mol l^{-1} octopamine solution in saline, saline alone as a control and ligated segments without injection. A digital SLR camera with an 18–55 mm lens was placed 40 cm above the wells containing the light organs to record bioluminescent output. Thirty-second exposures at maximum aperture were captured every 10 min using a Pclix time-lapse device (Pclix, version 2.2, Visual Effects Inc., Toronto, Ontario, Canada). As an additional treatment, carbon dioxide was released into a chamber containing the ligated and removed posterior segments and light output was noted.

To expose the isolated light organ to solutions, the light organ was dissected from a larva and placed into a glass cavity block filled

with 100 µl of phosphate-buffered saline (PBS) for 7 min. During dissection, all tracheal and neural tracts were disconnected from the light organ and the gut was removed. After 7 min, the bathing solution was replaced with a 0.58 mol l^{-1} amine solution or a gentle stream of carbon dioxide was directed into a chamber enclosing the cavity block. A digital SLR camera was focused on the cavity block and images were captured every minute for the 7 min preceding the treatment and the following 20 min. Identical exposure and lens zoom settings were used for all treatments. Each treatment was replicated at least five times.

Quantifying bioluminescence

Individual images (8 bit, 1024×768 pixels) were analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to calculate the number of pixels associated with each glowing larva. Images were converted to 8 bit grey scale and a threshold pixel value was established above which bioluminescence was distinguishable from background noise. The 'integrated density' function of ImageJ was used to calculate the light output intensity of each individual pixel constituting the light organ above the set threshold level for each frame. No change in spectral composition of bioluminescence was noticed after treatments; however, it was not specifically measured. Camera and lens settings and the distance between the subject and the camera were constant within each treatment (feeding, direct injection, ligation and injection, and bathing).

Statistical analysis was performed in the program R (version 2.9.2; http://www.r-project.org/). Multiple comparisons were made between treatments using the linear mixed effects (LME) model and ANOVA calculations, with time before and after feeding, and presence/absence or concentration of the biogenic amine as fixed factors. The LME model enables the integration of fixed and random effects, allowing larvae to be treated as individuals and pre- and post-feeding values and replicates to be incorporated into the statistical analysis (Lee and Nelder, 2001). Bioluminescence levels were compared between the control and treatment groups. Responses to treatments varied between individuals; consequently, the data appeared skewed in a linear plot. Data were transformed using the square root of the bioluminescence values. After transformation, the data included fewer extreme outliers allowing analysis using the LME model. A minimum of eight individuals were analysed for each treatment, except for the light organ bathing experiments where the minimum was five.

Immunohistochemistry

Arachnocampa flava larvae were dissected to expose the ventral nerve cord and light organ, and the tissue was immersed in 4% formaldehyde in PBS. After fixation for 10–15 min (serotonin immunostaining) or 30 min (acetylated α-tubulin immunostaining), the tissue was washed with PBS and then washed in PBS with 0.4% Triton X-100 and 0.25% bovine serum albumin (PBT) (Sigma-Aldrich) for 30 min. For acetylated tubulin immunostaining, tissue was incubated in PBT for 24 h. Tissue was pre-incubated in a blocking solution consisting of 98% PBT, 2% normal goat serum (Sigma-Aldrich) for 90 min.

Tissues were incubated in either rabbit polyclonal anti-serotonin primary antibody (ImmunoStar, Hudson, WI, USA) (1:500) or mouse monoclonal anti-acetylated α-tubulin primary antibody (Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia) (1:200) for 48 h at 4°C, and then incubated in secondary antibody, either anti-rabbit Alexa Fluor 488 (Invitrogen) for serotonin or anti-mouse Alexa Fluor 488 (Invitrogen) for acetylated tubulin. Secondary antibody solutions included a 1 µg ml^{-1} concentration of DAPI (Invitrogen) and AlexaFluor 568 phalloidin (Invitrogen) at a concentration of 6.6 µmol l^{-1}. Tissue was incubated in the secondary antibody at 4°C for 24 h. The tissue was rinsed in PBS four times over 60 min, cleared in 70% glycerol with 2% propyl gallate (Sigma-Aldrich) in PBS for 90 min and mounted onto a slide.

Tissue prepared for octopamine immunostaining, including the brain, the abdominal ganglia and the light organ, was fixed using a number of different protocols involving both glutaraldehyde and formaldehyde. Prior to dissection, glowworms were immobilised by cooling on ice. The dissection instruments that were used to prepare tissue were also placed on ice for 10–20 min to diminish the stress-induced release of octopamine to the haemolymph (Orchard et al., 1981). A mouse monoclonal anti-octopamine antibody (Jena Bioscience, Jena, Germany) and a rabbit polyclonal anti-octopamine (Millipore, Billerica, MA, USA) were used at a range of concentrations. A number of protocols based on reports using the same antibodies were trialled (Clark and Lange, 2003; Dacks et al., 2005; Certel et al., 2007; Busch et al., 2009) and were modified further after communication with some of the authors of these studies (S. Certel, H. Tanimoto and M. Selcho, personal communication), but were unsuccessful. Modifications included the use of different fixatives – formaldehyde, glutaraldehyde and 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride – and different periods of primary antibody infiltration including 12, 24, 48 and 72 h at 4°C. No fluorescent signal was obtainable from whole tissue stained using these techniques. Sensitivity to fixation methods and penetration of antibody into tissues were identified as common problems experienced in use of this antibody (H. Tanimoto, personal communication); however, it is also possible that the antibody does not cross-react with *Arachnocampa* octopamine.

Images were obtained using a Zeiss confocal laser-scanning microscope (LSM5 Pascal; Carl Zeiss Australasia, Sydney, New South Wales, Australia) using laser and filter settings appropriate for the fluorochromes of the secondary antibody, phalloidin and DAPI. To create images of the nervous system, the maximal intensity of pixels from each scanned section was used to create a single projection from an image stack of various depths. Images were adjusted for contrast and brightness.

Histological sections

Larvae were injected with fixative (4% formaldehyde in PBS) and the terminal region containing the light organ was cut from the rest of the body under fixative. The tissue was washed twice in PBS for 10 min and then dehydrated in an ethanol series (10% increments from 30 to 100%). Samples were then infiltrated with LR White resin (ProSciTech, Kirwan, Queensland, Australia), placed into 1 ml gelatine capsules and polymerised at 60°C for 24 h. Resin blocks were sectioned at 3 µm using a rotary microtome. Serial ribbons of sections were collected onto slides and stained using aqueous 0.3% Toluidine Blue and 5% boric acid.

RESULTS

Light organ anatomy and innervation

The four Malpighian tubules originate near the hindgut–midgut junction (Fig. 1). Four morphologically distinct sections are visible (Green, 1979b): (1) the free-lying hyaline region, (2) the free-lying pigmented region, (3) the cryptonephridial region, bound to the hindgut by connective tissue, and (4) the light organ itself (Fig. 1). Sections reveal that the cells of the light organ are large (50–100 µm in diameter) with a dense cytoplasm (Fig. 1). The reflector, composed of a compact mass of air-filled trachea, is opaque under transmitted

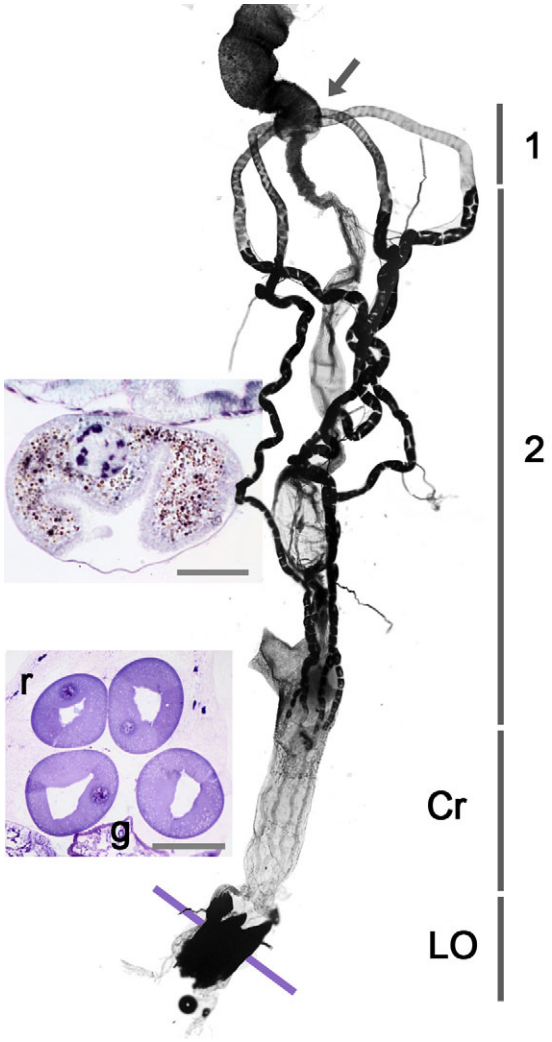

Fig. 1. The Malpighian tubules, cryptonephridial region and light organ of *Arachnocampa flava*. The four Malpighian tubules originate at the hindgut–midgut junction (arrow). The four regions defined by Green (Green, 1979b) are indicated as regions 1 and 2, the cryptonephridial region (Cr) and the light organ itself (LO). The light organ is opaque because of the light-scattering properties of the tracheal reflector. The top inset shows a section through a cryptonephridial region of a single Malpighian tubule (scale bar, 40 μm). The bottom inset is a section through the light organ, showing the four tubules in section; the reflector (r) lies atop the cells and the gut (g) below (scale bar, 100 μm).

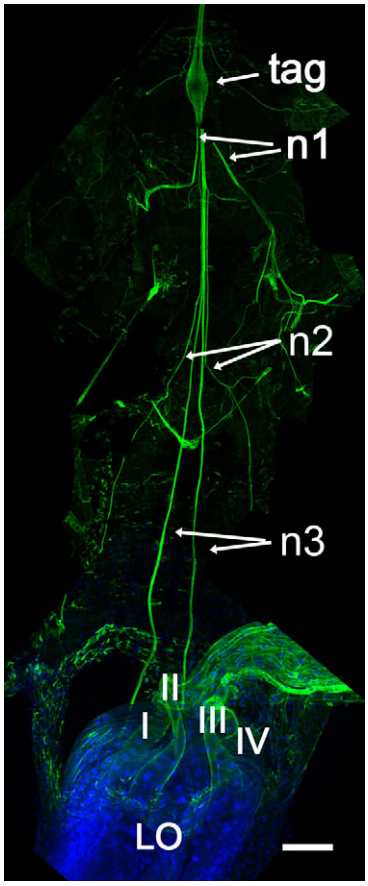

Fig. 2. Neuroanatomy of the terminal region of an *A. flava* larva, immunostained with an antibody against acetylated tubulin (green) and a nuclear stain (blue). Three pairs of nerves (n1, n2 and n3) arise from the terminal abdominal ganglion (tag). The third pair, n3, sends processes beneath the light organ (LO). The four pairs of Malpighian tubules (I, II, III and IV) are seen contributing to the light organ. Scale bar, 200 μm.

illumination. In sections, the reflector is closely apposed to the cells of the light organ.

An anti-acetylated α-tubulin antibody was used to trace nerves between the light organ and the terminal abdominal ganglion (TAG). From the TAG in the seventh abdominal segment, the median nerve continues posteriorly, progressively giving rise to paired branches. The three pairs of nerves originating posterior of the TAG were numbered according to their origin. Pairs 1 and 2 project laterally away from the median nerve to innervate body wall muscles (Fig. 2). Pair 3 projects ventrally underneath the light organ and then re-curves anteriorly and innervates the cells of the light organ from the posterior aspect by fine processes. The neural processes project throughout the tissue of the light organ, with several projecting horizontally across several Malpighian tubules. Neural processes exit the light organ *via* its anterior end and project onwards to innervate the gut. Some neural branches continue past the light organ posteriorly, innervating the anal papillae and rectal muscles.

Immunostaining with an anti-serotonin antibody revealed serotonin-immunoreactive nerve cell bodies and processes in the TAG and the more anterior abdominal ganglia. No serotonergic processes were observed projecting posteriorward from the TAG towards the light organ (data not shown). We were not able to detect octopamine in *A. flava* using immunostaining, even in the brain and thoracic ganglia where octopaminergic neurons would be expected to be detectable.

Influence of biogenic amines on bioluminescence

We trialled four methods of introducing biogenic amines: ingestion of prey items injected with test solution, bathing the dissected light organ in test solution, injection of solution into the whole larva and ligation of the terminal region followed by injection. To introduce test solutions *via* ingestion, individual *D. melanogaster* were injected with a defined volume and concentration of amine solution or saline and then fed to larvae at the onset of the dark period. The light output during the 12 h dark period was compared pre- and post-feeding. Control experiments showed elevated levels of bioluminescence through the night after feeding, confirming that feeding itself stimulates light output (Willis et al., 2011). Ingestion

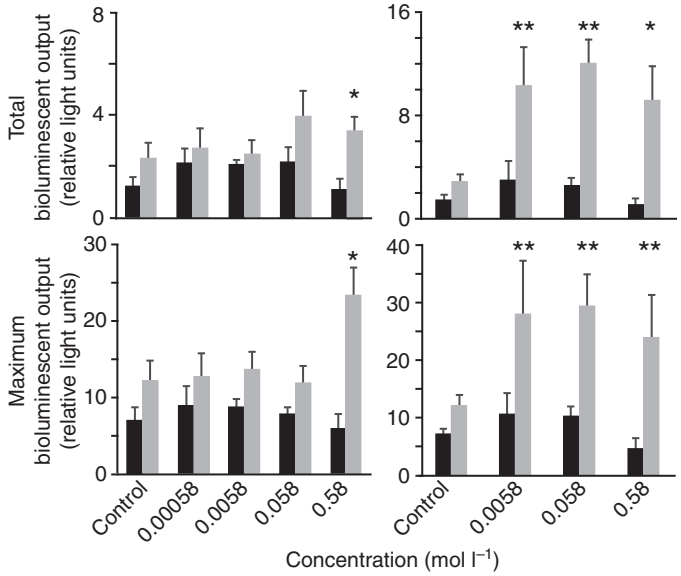

Fig. 3. Total and maximum bioluminescent output (means ± s.e.m.) of *A. flava* larvae after ingestion of *Drosophila melanogaster* flies injected with different concentrations of octopamine (left) and phentolamine (right). The paired comparisons show the night before exposure (black bars) compared with the night after exposure (grey bars). The control treatments show that *A. flava* larvae produce more light on the night after they have captured prey. Significant differences to the control are shown with asterisks (**P*<0.05; ***P*<0.001).

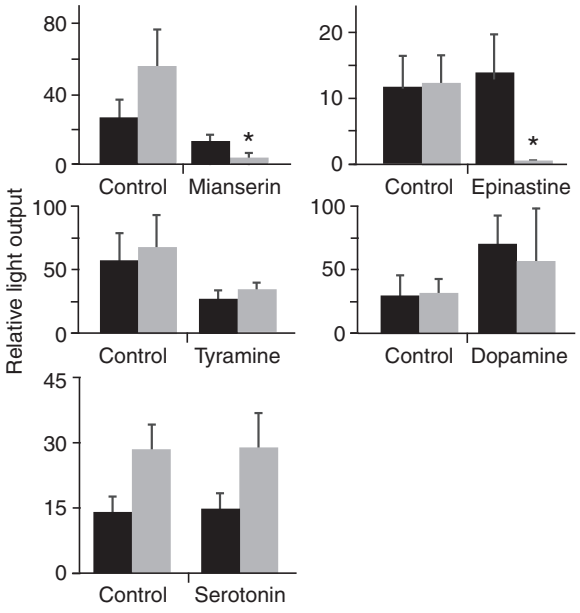

Fig. 4. Relative light output (means ± s.e.m.) of *A. flava* light larvae after ingestion of *D. melanogaster* flies injected with 1.2 µl of 0.58 µmol l^{-1} mianserin, epinastine, tyramine, dopamine or serotonin. The paired comparisons show the bioluminescence levels in the night before exposure (black bars) compared with the night after exposure (grey bars). Controls were fed flies injected with 1.2 µl of saline. A significant reduction in light output occurred on the night after ingestion of the antagonists mianserin and epinastine (**P*<0.05), whereas tyramine, dopamine and serotonin had no significant effect.

of octopamine and its antagonists – phentolamine, mianserin and epinastine – all affected bioluminescence output (Figs 3, 4). At 0.58 mol l^{-1}, octopamine produced a small (1.5-fold) increase compared with the control, whereas the antagonist phentolamine produced a much higher increase in bioluminescence, approximately four times more than the control (Fig. 3)

Octopamine and phentolamine were tested at lower concentrations to determine whether a dosage effect was apparent. Two features were compared, the total bioluminescent output and the maximum level of bioluminescence reached before and after feeding (Fig. 3). Octopamine produced a significant increase in total and maximum light output compared with the control only at the highest concentration tested. Phentolamine produced significant increases in both total and maximum light output at all concentrations.

Phentolamine ingestion also caused some larvae to continue to bioluminesce during the subsequent light period, a situation that does not occur naturally as ambient light inhibits glowing (Gatenby, 1959). This component of light output was not quantifiable because of the background light level but was not seen after treatment with any other antagonist using this technique. During the following night (the second night after treatment), phentolamine-fed larvae produced significantly less light output compared with controls (data not shown), whereas octopamine ingestion resulted in light output levels on the following night that were not statistically different from the pre-treatment level.

The feeding method was used to test two additional antagonists, mianserin and epinastine. Both compounds depressed, rather than elevated, larval light output (Fig. 4). Epinastine feeding resulted in larvae failing to bioluminesce at all (Fig. 4). The biogenic amines serotonin, tyramine and dopamine had no effect on bioluminescence when introduced using this procedure (Fig. 4).

Bathing of the dissected light organ in a saline solution containing the biogenic amines and antagonists was also utilised. The isolated light organ emits light upon dissection, the light diminishing progressively to undetectable levels after approximately 25 min (Fig. 5). Addition of octopamine to the bathing solution caused an immediate threefold to fourfold elevation in bioluminescence followed by a decline over approximately 13 min (Fig. 5). The antagonists phentolamine and epinastine produced complete dimming over 1 to 5 min. Mianserin produced a large increase in light output, peaking at approximately 13-fold the pre-treatment level, and still producing light 20 min after exposure (Fig. 5). Solutions of serotonin or dopamine produced no substantial difference in the bioluminescence production curve compared with the controls. Also, the octopamine precursor, tyramine, had no effect. Perfusion of carbon dioxide into the container holding the light organ in a saline bathing solution resulted in a fourfold increase in light output, returning to pre-exposure levels after 20 min (Fig. 5).

Direct injection of larvae with amine or antagonist solutions was trialled. The injection process itself is highly traumatic and invariably leads to death within hours. Control injections of saline resulted in no light output through the 12 h dark period following injection at the onset of darkness. Injection of a low concentration of octopamine resulted in some bioluminescence at levels well below the pre-injection level and a higher concentration produced a higher post-injection level of bioluminescence, although still below the pre-injection level. Because of the impact of the injection itself, the method was not used to assay for octopamine antagonists or for serotonin, tyramine or dopamine.

Ligation of the light organ and subsequent injection with solutions was tested as another method of administering biogenic amines. Upon ligation and separation of the light organ from the terminal abdominal ganglion, the light organ spontaneously produces bioluminescence.

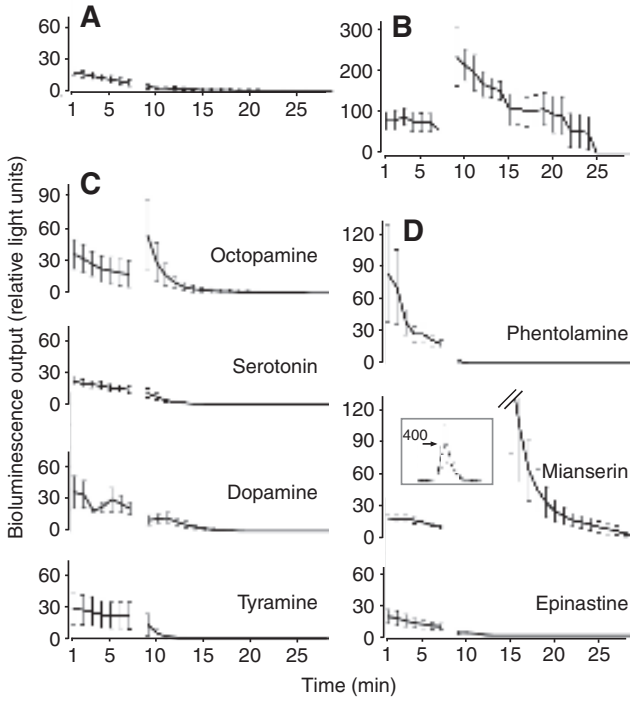

Fig. 5. Bioluminescence intensity (means ± s.e.m.) of *A. flava* light organs in (A) control conditions and upon exposure to (B) carbon dioxide, (C) biogenic amines and (D) octopamine antagonists. The dissected light organs were initially placed in a saline bath (time 1 on the *x*-axis) and the isolated light organ's bioluminescence output was imaged for 7 min, then the saline was replaced with the treatment compound dissolved in saline or, in the case of carbon dioxide, gas was introduced into the chamber. In the control, the bathing solution was replaced with more of the same. All treatments are presented at the same scale on the *y*-axis except for the carbon dioxide treatment. The high light output associated with mianserin treatment is depicted in the inset, with the peak value reaching approximately 400 units. Each treatment was replicated six times, except for the epinastine treatment (five replicates).

Injection of saline alone into the isolated ligated section resulted in a slight depression of bioluminescence compared with the uninjected control. Injection of octopamine resulted in an elevation of bioluminescent output but the variability in the response was such that the difference to the non-injected control was not significant. This method was not used to assay for octopamine antagonists or for serotonin, tyramine or dopamine. Exposure of the ligated light organ to carbon dioxide produced elevated bioluminescence.

DISCUSSION

Morphology and neuroanatomy of Malpighian tubules and the light organ

The four Malpighian tubules of *Arachnocampa* show several morphologically and functionally discrete regions (Green, 1979b), transitioning from a proximal excretory region lying free in the haemolymph to a cryptonephridial region lying closely bound to the hindgut and the light organ itself, which is backed by a reflector of densely packed trachea. No substantial differences were seen between *A. flava* and *A. luminosa*, so we assume that Green's comprehensive descriptions for *A. luminosa* are consistent across the genus. Within Diptera (true flies), possession of a cryptonephridium is now recognised as an autapomorphy of Keroplatidae, characteristic of all three subfamilies: the Keroplatinae, the Macrocerinae and the Arachnocampinae (Matile, 1990). We suggest that the ancestral function of the cryptonephridium is water conservation, a suggestion that was discounted by Green (Green, 1980) in the particular case of *Arachnocampa* because of the larva's hygrophilous nature. However, free water could be a limited resource in *Arachnocampa*: first, larval immobility and confinement to the snare in sheltered environments means that larvae may not have access to water droplets for ingestion; second, the carnivorous habit means that food is sporadically available and nutrient-rich but water-poor; and third, the production of large amounts of mucus and use of mucous droplets to capture prey exposes larvae to water loss through evaporation. *Arachnocampa* shows some water-conserving adaptations: larvae re-ingest droplets from their fishing lines as they carry out web maintenance (D.J.M., unpublished observations); also, the mucous droplets may be hygroscopic (Stringer, 1967), allowing the extraction of atmospheric water. The cryptonephridium might then be a water-conserving adaptation in the group just as it is in Coleoptera (Grimstone et al., 1968). Given that the cryptonephridium is a plesiomorphism, the light organ in *Arachnocampa* is more likely to be evolutionarily derived from cells of a cryptonephridial system than from purely excretory cells. Perhaps high metabolic activity in these cells pre-adapted them to light production, as it has been postulated that the precursor for the evolution of bioluminescence signals is very low light emission associated with the activity of cellular oxygenases (Seliger, 1975; Hastings, 1983).

Bearing in mind that the bioluminescence systems of fireflies, krill and *Arachnocampa* are independently evolved, the pre-existing innervation of the organ or tissue could be drawn into a bioluminescence regulatory function; for example, the nerves innervating firefly light organs appear to be derived from spiracular nerves (Ghiradella and Schmidt, 2004). Using immunostaining, we observed fine terminal processes projecting from the third branch of the median connective nerve that arborise over the cells of the light organ. These are most likely the nerves observed by transmission electron microscopy directly innervating the photocytes of the light organ in *A. luminosa* (Green, 1979a). Some processes project horizontally across the midline, contacting all four tubules whose terminal cells constitute the light organ.

Serotonin immunoreactivity was observed in the terminal abdominal ganglion and in the transverse nerves anterior to the terminal and sixth abdominal ganglion, but no serotonin immunoreactivity was identified in the nerves posterior to the terminal abdominal ganglion, including those innervating the light organ. In addition, none of the serotonin introduction methods affected bioluminescence, indicating that serotonin plays little to no role in either high-level or direct regulation of light output. Given our inability to locate octopaminergic neurons, the neuroanatomical approach has not been able to inform whether octopamine is a neurotransmitter directly released from nerve terminals innervating the light organ as seen in the firefly (Copeland and Robertson, 1982).

Octopamine and antagonists

We developed a novel method of biogenic amine introduction based upon feeding *Arachnocampa* larvae live prey items (vinegar flies) that had been injected with amine solution allowing controlled doses without disturbance to the organism beyond food consumption. The bioluminescence arousal associated with prey capture (Stringer, 1967; Merritt and Aotani, 2008; Willis et al., 2011) was revealed in the controls, where feeding larvae uninjected or saline-injected flies consistently produced a twofold increase in bioluminescence output on the night following feeding. Octopamine feeding produced

an irregular pattern of light output with short-term, high-level peaks in comparison to the control. When a graded concentration series was administered, the irregular and increased light output was seen only at the highest concentration. Alternative methods of introducing octopamine produced similar outcomes. Whole-larva injection produced elevated bioluminescence levels compared with the controls. Injection into a ligated posterior section containing the light organ also produced elevated bioluminescence. The effect can be narrowed down to the light organ isolated from the terminal abdominal ganglion, as bathing of isolated light organs in octopamine solution produced threefold to fourfold elevated light levels. Tyramine, dopamine and serotonin had no effect on bioluminescence using any of the introduction methods, allowing us to eliminate their role in regulating light production.

The foregoing results indicate that octopamine is associated with the release or regulation of bioluminescence. However, treatment with octopamine antagonists does not necessarily produce the opposite effect to octopamine, i.e. blocking bioluminescence. They produced different results depending on the compound tested and whether it was introduced by feeding or light organ bathing. Epinastine, regarded as a high-affinity, highly specific antagonist of octopamine (Roeder et al., 1998), depressed light output after ingestion and when bathing the light organ, supporting its role as an antagonist of octopamine. Phentolamine, used in many studies as an octopamine receptor antagonist (Lafon-Cazal et al., 1987; Stevenson et al., 1992; Meyer-Fernandes et al., 2000; Cohen et al., 2002) caused a marked – up to fivefold – increase in total light output through the night following oral introduction. The effect is long lasting, persisting into the following photophase, as larvae do not show the normal light-induced dousing response to external light (Gatenby, 1959; Meyer-Rochow and Waldvogel, 1979). Bathing of the isolated light organ in phentolamine had the opposite effect to its ingestion: bioluminescence was depressed. Mianserin, also a high-affinity antagonist for octopamine (Roeder et al., 1998), had no effect when ingested; however, it caused an extremely high bioluminescent output in the light organ bath. Mianserin is also known to block serotonin uptake (Evans, 1985), so its activity upon the isolated light organ could indicate a possible indirect role for serotonin in regulating bioluminescence.

Together, the effects of octopamine antagonists are difficult to reconcile with the effect of octopamine itself. As a group, the different antagonists would be expected to have the same effect on light production and produce the opposite response to octopamine. One caveat to bear in mind is that the octopamine antagonists can cross-react with other receptor types at certain concentrations (Roeder et al., 1998; Cohen et al., 2002), possibly leading to variable and non-specific results. Second, considering the ingestion experiments, the ability of the different compounds to cross from the gut into the haemolymph could differ. Third, ingestion of octopamine and its antagonists could impact on the full nervous system, affecting the larva's general state of arousal upstream of the light organ effector system. The marked, long-lasting elevation of bioluminescence after consumption of phentolamine indicates that it may induce the release of a product from the digestive system or affect a process otherwise linked to feeding that de-regulates bioluminescence suppression, similar to the role of anaesthetics.

Bathing of the light organ itself is the most informative procedure in revealing the effects of biogenic amines and antagonists on the cells of the light organ and the associated tracheal system, if indeed the tracheal system is involved in regulating bioluminescence. In bathing experiments, octopamine consistently elevates light levels. Phentolamine and epinastine have little effect but there are signs that they suppress light output, supporting their role as octopamine antagonists. Mianserin produces a very high light output similar to or greater than levels seen under anaesthesia. Carbon dioxide dissolved in the bathing solution causes a release of light, as does whole-animal anaesthesia with carbon dioxide or ether (Lee, 1976), or carbon dioxide anaesthesia of a ligated section containing the light organ only (present study). Carbon dioxide is thought to block synaptic transmission at the neuromuscular junctions (Badre et al., 2005), opening the possibility that muscle control is required to restrict bioluminescent output. We saw no obvious muscle sphincters associated with the light organ or its tracheal supply, but this avenue is worthy of further investigation.

Prior to this study, the existing model for regulation of bioluminescence in *Arachnocampa*, based on use of anaesthetics (Lee, 1976) and ligations (Gatenby, 1959), was that bioluminescence is actively repressed during the non-glowing phase and the repression is partially released during the bioluminescence phase. The effect of the anaesthetic, carbon dioxide, on the isolated light organ from the present study indicates that the repression is at least partially mediated at the light organ itself. Removal of the central nervous sytem through ligation leads to loss of control over bioluminescence, but our experiments show that light is emitted at relatively low levels compared with exposure to anaesthetic. Experimental exposure to octopamine suggests that it is involved in the regulation of repression, either by acting directly on the cells of the light organ or through regulation of oxygen access to the cells. Perhaps octopamine functions at the light organ to modulate the light output at an intermediate level at night during the glowing cycle. Administration of the antagonists phentolamine or mianserin blocks the octopamine receptors and results in very high bioluminescent output levels, similar to the effect of anaesthetics, but, of the pair, only mianserin acts directly on the light organ. The results reinforce the intimate interaction between feeding and bioluminescence in *Arachnocampa*. The act of feeding results in elevated bioluminescence in both the short term (present study) and the long term (Willis et al., 2011). At least a component of that control may be attributable to the biogenic amine octopamine.

ACKNOWLEDGEMENTS

We thank Simon Blomberg for statistical advice, and members of the Merritt laboratory, particularly Rebecca Morley and Robyn Willis, for help and support.

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